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(11)

**EP 1 103 617 A2**

(12)

**EUROPEAN PATENT APPLICATION**

(43) Date of publication:  
**30.05.2001 Bulletin 2001/22**

(51) Int Cl.7: **C12N 15/82, C12N 15/54,  
C12N 9/10, A01H 5/00**

(21) Application number: **01100504.8**

(22) Date of filing: **11.02.1992**

(84) Designated Contracting States:  
**AT BE CH DE DK ES FR GB GR IT LI LU MC NL SE**

(30) Priority: **13.02.1991 DE 4104782**

(62) Document number(s) of the earlier application(s) in  
accordance with Art. 76 EPC:  
**92904007.9 / 0 571 427**

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yet been filed**

Remarks:

This application was filed on 09 - 01 - 2001 as a  
divisional application to the application mentioned  
under INID code 62.

(54) **Plasmids containing DNA-sequences that cause changes in the carbohydrate composition  
in plants, as well as plant cells and plants containing these plasmids**

(57) Plasmids are described having DNA sequences that after insertion into the genome of the plants cause changes in the carbohydrate concentration and the carbohydrate composition in regenerated plants.

These changes can be obtained from a sequence of a branching enzyme that is located on these plasmids. This branching enzyme alters the amylose/amylopectin ratio in starch of the plants, especially in commercially used plants.

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## Description

[0001] The present invention relates to plasmids containing DNA-sequences which contain information that, after insertion into a plant genome, cause changes in the carbohydrate concentration and the carbohydrate composition in regenerated plants, as well as plant cells and plants containing sequences from these plasmids.

[0002] Because of the continual growth in world population, there is a continually growing demand for nutrient and raw materials. It is the task of biotechnological research to achieve a change of the content as well as yield of crops. To do this the metabolism of the plants has to be altered.

[0003] A particular interest is the possibility of using plant ingredients as renewable raw material sources e.g. for the chemical industry. This is especially of great importance for two reasons. Firstly, up to now, mineral oil and coal deposits have been the main source of raw materials for the petrochemical industry but these deposits are finite and it can be seen that alternative, renewable raw material sources must be developed.

[0004] Secondly, the present situation of agriculture in Europe and North America has lead to a surplus of crops grown for their nutritive properties. This causes obvious financial and political problems in agriculture. Alternative products for which there is a higher quantitative demand could be a solution to this problem.

[0005] Renewable raw materials can be divided into fats and oils, proteins and carbohydrates, such as mono-, di-, oligo- and polysaccharides. The most important polysaccharides are starch and cellulose. In the EEC, the total starch production in 1987-1988 comprised maize (60%), wheat (19%) and potato (21%).

[0006] For an increasing use of plant starch as an industrial raw material the quality of the starch must meet the demands of the processing industry. Important considerations include the amylose to amylopectin ratio, the chain length, the branching grade of the amylopectin as well as the size of the starch granules.

[0007] The main biochemical synthetic pathways for the production of starch in higher plants are well known. Starch consists of amylose and amylopectin, in which the amylose consists of a linear  $\alpha$ -1,4-glucan and amylopectin consists of  $\alpha$ -1,4-glucans, which are connected to each other via  $\alpha$  1,6-linkages and thus form a branched polyglucan. The so-called branching enzyme (Q-enzyme) is responsible for the introduction of the  $\alpha$ -1,6-linkage. One method for the production of starch which only has a linear  $\alpha$ -1,4-glucan structure is therefore by the inhibition of the enzymatic activity of the proteins and/or the inhibition of the biosynthesis of the branching enzyme. New biotechnology processes for the genetic alteration of dicotyledonous and monocotyledonous plants by transfer and stable installation of single isolated genes or groups of genes are known (Gasser and Fraley, Science 244, 1293-1299). The possibility of specific expression of foreign genes inserted in the plant by gene technology, primarily in potato tubers, is also known (EP 375092 and Rocha-Sosa et al., EMBO J. 8, 23-29 (1989)).

[0008] The present invention provides plasmids containing DNA-sequences which contain information that, after insertion into a plant genome, cause changes in the carbohydrate concentration and the carbohydrate composition in regenerated plants.

[0009] The invention further provides plant cells containing sequences from these plasmids which can be regenerated to whole plants, as well as plants containing sequences from these plasmids.

[0010] The term "plant" means a commercially useful plant, preferably maize, barley, wheat, rice, peas, soya beans, sugar cane, sugar beet, tomato, potato or tobacco.

[0011] Carbohydrates which can be altered by the DNA sequences are mono-, di-, oligo- or polysaccharides. Starch is an example of a polysaccharide which can be modified in plants and plant cells.

[0012] With the plasmids of the invention, it is possible to modify the amylose to amylopectin ratio of the starch in plant cells and in plants. This is possible through the presence of a branching enzyme, located on the plasmid, which has the following sequence (Seq ID No.1):

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	10	20	30	40	50	60
1	TCAGGAGC	GGTCTT	GGGATATT	TCTTCCAC	CCCCAAA	ATCAAGAGTTAGAAAAGATGAAAG
5	61	GATGAAGC	CACAGTT	CAGCTATT	TCCTGTTT	GACCGATGACAATTCGACAATGGCACC
	121	CCTAGAGG	AAGATGT	CAACACT	GAAAAAT	ATTGGCCTCCTAAATTTGGATCCAACTTTGG
	181	ACCTTAT	CTAGAT	CACTTC	CAGACAC	AGAATGAAGAATATGTGGATCAGAAAAATGCTCAT
10	241	TGAAAAA	TATGAGG	GACCCCT	TGAGGA	ATTTGCTCAAGGTTATTTAAAAATTTGGATTCAA
	301	CAGGGA	AAGATG	GTTCAT	AGTCTAT	CGTGAATGGGCTCCTGCTGCTCA6GAAGCAGAAGT
	361	TATTGG	CGATTT	CAATGG	TAGGAAC	GGTTCTAACCACATGATGGAGAAGGACCAATTTGG
	421	TGTTT	GGAGT	ATTAGA	ATTCCT	GATGTTGACAGTAAGCCAGTCATTCCACACAACCTCCAG
15	481	AGTTA	AGTTT	CGTTT	CAAAC	ATGGTAATGGAGTGTGGGTAGATCCTATCCCTGCTTGGAT
	541	AAAGT	ATGCC	ACTG	CAGAC	GCCACAAAGTTTGCAGCACCATATGATGGTGTCTACTGGGA
	601	CCCAC	CACCTT	CAGAA	GGTACC	ACTTCAAATACCCTCGCCCTCCCAAACCCGAGCCCC
20	661	ACGAAT	CTACG	AAGCAC	ATGTC	GGCATGAGCAGCTCTGAGCCACGTGTAAATTCGTATCG
	721	TGA	TTTTG	CAGAT	GATG	TTTTACCTCGGATTAAAGGCAAATAACTATAATACTGTCCAATT

781 GATGGCCATAATGGAACATTCTTACTATGGATCATTGGATATCATGTTACAACTTTTT  
 841 T6CTGT6AGCAATAGATATG6AAAACCCGGAGGACCTAAAGTATCTGATAGATAAAGCACA  
 5 901 TAGCTTGGGTTTACAG6TTCTG6TGGATGTA6TTACAGTCATGCAA6CAATAAT6TCAC  
 961 T6ATGGCCTCAAT6GCTTTGATATTGGCCAAGGTTCTCAAGAATCCTACTTTCAT6CTG6  
 1021 AGAGCGAGGGTACCATAAGTT6TGGGATAGCA66CT6TTCAACTAT6CCAATTGGGAGGT  
 1081 TCTTCGTTTCTTCTTTCCAACTTGAGGTGGTGGCTAGAAGAGTATAACTTTGACGGATT  
 10 1141 TCGATTTGATGGAATAACTTCTATGCTGTAT6TTCATCATGGAATCAATATGGGATTTAC  
 1201 AGGAACTATAATGAGTATTTCA6CGAGGCTACAGATGTT6ATGCTGTGGTCTATTTAAT  
 1261 GTTGGCCAATAATCTGATTCACAAGATTTTCCAGACGCAACTGTTATTGCCGAAGATGT  
 15 1321 TTCTGGTATGCCGGGCTTAGCCGGCCTGTTTCTGAGGGAGGAATTG6TTTTGATTACCG  
 1381 CCTGGCAATG6CAATCCAGATAAGTGGATAGATTATTTAAAGAATAAGAATGATGAAGA  
 1441 TTGGTCCATGAAGGAA6TAACATCGAGTTTGACAAATAGGAGATATACAGAGAAGTGTAT  
 20 1501 AGCATATGCGGAGAGCCATGATCAGTCTATTGTCGGTGACAAGACCATTGCATTTCTCCT  
 1561 AATGAACAAAGAGATGTATTCTGGCATGTCTTGCTTGACAGATGCTTCTCCTGTT6TTGA  
 1621 TGCAGGAATTGCGCTTGACAAGATGATCCATTTTTTTCACAATGGCCTTGGGAGGAGAGG  
 25 1681 GGTACCTCAATTTCATG66TAACGAGTTTGGCCATCCTGAGT66ATTGACTTCCCTAGTG  
 1741 AGGGCAATAATTGGAGTTATGACAAAT6TAGACGCCAGT6GAACCTCGCAGATAGC6AAC  
 1801 ACTTGAGATACAA6TTTATGAATGCATTTGATAGAGCTATGAATTGCTCGATGAAAA6T  
 1861 TCTCATTCCTCGCATCA66AAAACAGATAGTAA6CA6CATGGATGATGATAATAA66TTG  
 30 1921 TTGT6TTTGAAC6TGGT6ACCTGGTATTTGTATTCAACTTCCACCCAAATAACACATACG  
 1981 AAGGGTATAAAGTTGGATGTGACTTGCCAGGGAA6TACAGAGTTGCACTGGACAGT6ATG  
 2041 CTTGGGAATTT6GTGGCATGGAAGA6CTGGTCATGATGTTGACCATTTACATCACCAG  
 35 2101 AAGGAATACCTGGAGTTCCAGAAAACAAATTTCAATGGTCGTCCAAATTCCTTCAAAGTGC  
 2161 TGTCTCCTGCG6CAACAT6TGTGGCTTATTACAGAGTTGATGAACGCAT6TCATAAACTG  
 2221 AAGATTACCAGACAGACATTT6TAGTGAGCTACTACCAACAGCCAATATCGAGGAAAAGTG  
 40 2281 ACGA6AAACTTAAAGATTCATCATCTACAAATATCAGTACATCATCTACAAAAAAT6CTT  
 2341 ATTACAGAGTTGATGAACGCAT6TCAGAAGCTGAAGATTACCAGACAGACATTT6TAGTG  
 2401 AGCTACTACTACCAACA6CCAATATCGA6GAGA6TGACGAGAAACTT6ATGATTCAATAT  
 2461 CTACAAATATCAGTAACATTGGTCAGACTGTTGTAGTTTCTGTTGAGGAGAGAGACAAGG  
 45 2521 AACTTAAAGATTCACCATCTGTAAGCATCATTAGTGATGCTGTTCCAGCTGAAT666CTG  
 2581 ATTC6GATGCAAAC6TCTGGGGT6A66ACTAGTCAGATGATTGATC6ATCCTTCTACGTT  
 2641 GGTGATCTCGGTCGTCATGATGTCCTCAGGGTGGTAGCATTGACTGATTGCATCATAG  
 50 2701 TTTTTTTTTTTTTTTTTTAA6TATTTCTCTATG6CATATTATTAGCATCCAATAAATTTAC  
 2761 TGGTTGTTGATACATAGAAAAAGTGCATTTG6CAT6TATGTTTCTCTGAAATTTCCCCA  
 2821 GTTTTGGT6CTTT6CCTTTGGAGCCAA6TCTCTATATGTAATAAGAAAACTAAGAACAAT  
 55 2881 CACATATATAAAATGTTAGTAGATTACCA .

[0013] The property of the branching enzyme to modify the amylose/amylopectin ratio in starch is not limited to a

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coding sequence exactly as it is shown here but can also be represented by slightly different nucleotid sequences. The property of the branching enzyme is also not changed when the plasmids containing the branching enzyme, are modified in the plant cell or the plant.

**[0014]** To be active, the DNA sequence of the branching enzyme is fused to the regulatory sequences of other genes which guarantee a transcription of the DNA (coding) sequence of the branching enzyme. The DNA sequence can also be fused in an inverted direction to the regulatory sequences of other genes, whereby the 3'-end of the coding sequence is fused to the 3'-end of the promoter and the 5'-end of the coding sequence is fused to the 5'-end of the termination signal. In this way an anti-sense RNA of the branching enzyme is produced in the plant. The regulatory sequences are hereby promoters and termination signals of plant or viral genes, such as for example the promoter of the 35S RNA of the cauliflower mosaic virus or the promoter of the class I patatin-gene B 33 and the termination signal of the 3'-end of the octopine synthase gene of the T-DNA of the Ti-plasmid pTiACH5.

**[0015]** Plant cells containing sequences from these plasmids can be regenerated in known manner to complete transgenic plants. It is possible to insert simultaneously, more than one copy of these sequences into a plant cell or plant.

**[0016]** The following plasmids were deposited at the Deutsche Sammlung von Mikroorganismen (DSM) in Braunschweig, Germany on the 20th August 1990 (deposit number):

Plasmid	P35 S-BE	(DSM 6143)
Plasmid	P35 S-anti-BE	(DSM 6144)
Plasmid	P33-BE	(DSM 6145)
Plasmid	P33-anti-BE	(DSM 6146)

### Description of the Figures

**[0017]** Figure 1 shows the restriction map of the 13.6 kb plasmid P35 S-BE. The plasmid contains the following fragments.

A =	Fragment A (529 bp) contains the 35S promoter of the cauliflower mosaic virus (CaMV). The fragment contains the nucleotides 6909-7437 of the cauliflower mosaic virus.
B =	Fragment B (2909 bp) contains the DNA fragment which codes for the branching enzyme.
C =	Fragment C (192 bp) contains the polyadenylation signal of the gene 3 of the T-DNA of the Ti-plasmid pTiACH5 from the nucleotide 11749 to 11939.

**[0018]** Also shown are the cleavage sites described in Example 1.

**[0019]** Figure 2 shows the restriction map of the 13.6 kb plasmid P35 S-anti-BE. The plasmid contains the following fragments:

A =	Fragment A (529 bp) contains the 35S promoter of the cauliflower mosaic virus (CaMV). The fragment contains the nucleotides 6909 to 7437 of the CaMV.
B =	Fragment B (2909 bp) contains the DNA fragment which codes for the branching enzyme.
C =	Fragment C (192 bp) contains the polyadenylation signal of gene 3 of the T-DNA of the Ti-plasmid pTiACH5. The fragment contains the nucleotides 11749-11939.

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[0020] Also shown are the cleavage sites described in Example 2.

[0021] Figure 3 shows the restriction map of the 14.6 kb plasmid P33-BE. The plasmid contains the following fragments.

A =	Fragment A (1526 bp) contains the Dral-Dral-fragment of the promoter region of the patatin-gene B33. The fragment contains the nucleotide positions -1512 to +14.
B =	Fragment B (2909 bp) contains the DNA fragment which codes for the branching enzyme.
C =	Fragment C (192 bp) contains the polyadenylation signal of the gene 3 of the T-DNA of the Ti-plasmid pTiACH5. The fragment contains the nucleotide positions 11749-11939.

[0022] Also shown are the cleavage sites described in Example 3.

[0023] Figure 4 shows the restriction map of the 14.6 plasmid P33-anti-BE. Plasmid contains the following fragments:

A	Fragment A (1526 bp) contains the Dral-Dral fragment of the promoter region of the patatin gene B33. The fragment contains the nucleotide position -1512 to +14.
B =	Fragment B (2909 bp) contains the cDNA-fragment which codes for the branching enzyme.
C =	Fragment C (192 bp) contains the polyadenylation signal of the gene 3 of the T-DNA of the Ti-plasmid pTiACH5. The fragment contains the nucleotides 11749-11939.

[0024] Also shown are the cleavage sites described in Example 4.

[0025] In order to understand the examples forming the basis of this invention all the processes necessary for these tests and which are known per se will first of all be listed:

### 1. Cloning process

[0026] The vectors pUC18/19 and pUC118, and the M13mp10 series (Yanisch-Perron *et al.*, Gene (1985), 33, 103-119) were used for cloning.

[0027] For plant transformation, the gene constructions were cloned into the binary vector BIN19 (Bevan, Nucl. Acids Res. (1984), 12, 8711-8720).

### 2. Bacterial strains

[0028] The *E. coli* strain BMH71-18 (Messing *et al.*, Proc. Natl. Acad. Sci. USA (1977), 24, 6342-6346) or TB1 was used for the pUC and M13 mP vectors.

[0029] For the vector BIN19 exclusively the *E. coli* strain TB1 was used. TB1 is a recombinant-negative, tetracycline-resistant derivative of strain JM101 (Yanisch-Perron *et al.*, Gene (1985), 33, 103-119). The genotype of the TB1 strain is (Bart Barrel, personal communication): F'(traD36, proAB, lacI, lacZΔM15), Δ(lac, pro), SupE, this, recA, Sr1::Tn10 (TcR).

[0030] The transformation of the plasmids into the potato plants was carried out by means of the *Agrobacterium tumefaciens* strain LBA4404 (Bevan, M., Nucl. Acids Res. 12, 8711-8721, (1984); BIN19 derivative).

### 3. Transformation of *Agrobacterium tumefaciens*

[0031] In the case of BIN19 derivatives, the insertion of the DNA into the agrobacteria was effected by direct transformation in accordance with the method developed by Holsters *et al.*, (Mol. Gen. Genet. (1978), 163, 181-187). The plasmid DNA of transformed agrobacteria was isolated in accordance with the method developed by Birnboim and Doly (Nucl. Acids Res. (1979), 7, 1513-1523) and was separated by gel electrophoresis after suitable restriction cleavage.

### 4. Plant transformation

[0032] 10 small leaves, wounded with a scalpel, of a sterile potato culture were placed in 10 ml of MS medium with 2 % sucrose containing from 30 to 50 µl of an *Agrobacterium tumefaciens* overnight culture grown under selection. After from 3 to 5 minutes gentle shaking, the Petri dishes were incubated in the dark at 25°C. After 2 days, the leaves were laid out on MS medium with 1.6 % glucose, 2 mg/l of zeatin ribose, 0.02 mg/l of naphthylacetic acid, 0.02 mg/l of gibberellic acid, 500 mg/l of claforan, 50 mg/l of kanamycin and 0.8 % Bacto agar. After incubation for one week at 25°C and 3000 lux, the claforan concentration in the medium was reduced by half. The regeneration and cultivation of the plants were carried out according to known processes (Rocha-Sosa *et al* EMBO Journal 8, 23-29 (1989).

### 5. Analysis of genomic DNA from transgenic potato plants

[0033] The isolation of genomic plant DNA was effected in accordance with Rogers and Bendich (Plant Mol. Biol. (1985), 5, 69-76).

[0034] For the DNA analysis, after suitable restriction cleavage, 10 to 20 µg of DNA were analysed by means of Southern blots for the integration of the DNA sequences to be investigated.

### 6. Analysis of the total RNA from transgenic potato plants

[0035] The isolation of plant total RNA was carried out in accordance with Logemann *et al.* (Analytical Biochem. (1987), 163, 16-20).

[0036] For the analysis, 50 µg portions of total RNA were investigated by means of Northern blots for the presence of the transcripts sought.

### 7. Protein extraction

[0037] For the extraction of total protein from plant tissue, pieces of tissue were homogenised in protein extraction buffer (25 mM sodium phosphate pH 7.0, 2 mM sodium hydrogen sulphite), with the addition of 0.1 % (w/v) of insoluble polyvinylpyrrolidone (PVP).

[0038] After filtration through cellulose, cell detritus was centrifuged off for 20 minutes at 10,000 revolutions per minute and the protein concentration of the supernatant was determined in accordance with the method developed by Bradford (Anal. Biochem. (1976) 72, 248-254).

### 8. Detection of foreign proteins by means of immunological processes (Western blot)

[0039] The protein extracts were separated according to molecular weight by means of gel electrophoresis in SDS-PAGE (sodium dodecylsulphate polyacrylamide) gels. After SDS-PAGE the protein gels were equilibrated for from 15 to 30 minutes in transfer buffer for graphite electrodes (48 g/l of tris, 39 g/l of glycine, 0.0375 % SDS, 20 % methanol) and then transferred in a cooling chamber to a nitrocellulose filter and separated at 1.3 mA/cm<sup>2</sup> for from 1 to 2 hours. The filter was saturated for 30 minutes with 3 % gelatin in TBS buffer (20 mM tris/HCl pH 7.5, 500 mM NaCl), and the filter was then incubated for 2 hours with the appropriate antiserum in a suitable dilution (1:1000 - 10000 in TBS buffer) at room temperature. The filter was then washed for 15 minutes each with TBS, TTBS (TBS buffer with 0.1% polyoxyethylene-(20)-sorbitan monolaurate) and TBS buffer. After being washed, the filter was incubated for 1 hour at room temperature with alkaline phosphatase-conjugated goat-anti-rabbit (GAR) antibodies (1:7500 in TBS). The filter was then washed as described above and equilibrated in AP buffer (100 mM tris/HCl pH 9.5, 100 mM NaCl, 5 mM MgCl<sub>2</sub>). The alkaline phosphatase reaction was started by means of the substrate addition of 70 µl of 4-nitroterrazolium (NBT) solution (50 mg/ml of NBT in 70 % dimethyl-formamide) and 35 µl of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (50 mg/ml BCIP in dimethylformamide) in 50 ml of AP buffer. As a rule the first signals were observed after 5 minutes.

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### 9. Determination of the amylose/amylopectin ratio in starch of transgenic potato plants.

5 [0040] Leaf pieces, having a diameter of 10 mm were floated in 6% sucrose solution under continuous light for 14 hours. This light incubation induced a strong increased starch formation in the leaf pieces. After incubation, the amylose and amylopectin concentration was determined according to Hovenkamp-Hermelink et al (Potato Research 31, 241-246 (1988)).

10 [0041] The following examples illustrate the preparation of the plasmids according to the invention, the insertion of sequences from those plasmids into the plant cell as well regeneration of transgenic plants and the analysis of those transgenic plants.

#### Example 1

#### Preparation of the plasmid P35s-Be and insertion of the plasmid into the plant genome of the potato.

15 [0042] From a cDNA library in the expression vector  $\lambda$ gt11, different clones were identified that cross-react with an antibody that is directed against the branching enzyme of potatoes. These clones were used to identify complete clones from a cDNA library in the HindII-position the vector pUC 19 that originate from isolated mRNA of growing potato tubers. One clone isolated in this manner had an insert size of 2909 bp of the sequence:



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	10	20	30	40	50	60	
1	TCAGGA	GC66T	CCTTGG	6ATATTT	CTTCCAC	CCCCAAA	ATCAA
5	61	GATGAA	GCACAG	TTCA6C	TATTTCC	GCTGTTT	GACCGAT
	121	CCTAGAG	GAAAGAT	GTCAAC	ACTGAAA	AATATTG	6CCTCCT
	181	ACCTTAT	CTAGAT	CACTTCA	GACACAG	AATGAA	GA6ATAT
10	241	TGAAAA	ATATGAG	GGACCC	CTTGA	AGGAATTT	GCTCAAG
	301	CAGGGA	AGATGG	TTGCAT	AGTCTAT	CGTGAAT	GG6CTC
	361	TATTGG	CGATTT	CAATGG	TAGGA	ACG6TT	CTAACC
15	421	TGTTTG	GAGTAT	TAGAAT	TCTGAT	GTTGAC	AGTAA
	481	AGTTAA	GTTTT	CAACAT	GGTAAT	GGAGT	GT66G
	541	AAAGTAT	GCCACT	GCA6AC	GCCACA	AAAGTT	G6CAGC
	601	CCCACC	ACCTTC	GAAA	GGTACC	ACTTCA	AAATACC
20	661	ACGAAT	CTACGA	AGCACAT	GTG6GC	ATGAGC	AGCTCT
	721	TGAGTTT	G6CAGAT	6ATGTTT	ACCTCG	6ATTA	AGGCAA
	781	GATG6C	CATAAT	GGAACAT	TCTTACT	ATG6AT	CATTTG
25	841	T6CTGT	GAGCAAT	AGATAT	G6AAA	CCCGG	AGGACCT
	901	TAGCTT	G66TTT	ACAG6T	TCTGG	T66AT	GTAGTT
	961	TGATGG	CCTCAAT	G6CTTT	GATATT	G6CCA	AG6TT
30	1021	AGAGCG	AG6GT	ACCATA	AGTTGT	G6GATA	6CAGG
	1081	TCTTCG	TTTTCT	TTTCCA	ACTTG	AGGTGG	T6GCTA
	1141	TCGATTT	GATGGA	ATAACT	TCTATG	CTGTAT	GTTTCA
35	1201	AGGAA	ACTATA	ATGAGT	ATTTCA	GC6AGG	CTACAG
	1261	6TTG6C	CAATAA	TCTGAT	TCAAG	ATTTT	CCCAG
	1321	TTCTGG	TATG6C	GG6CCT	TAG6C	GGCCTG	TTTCTG
	1381	CCTG6C	AATG6C	AATCCC	CAGATA	AGTGG	ATAGAT
40	1441	TTGGT	CCATGA	AGGA	AGTAAC	ATCGAG	TTTGAC
	1501	AGCATAT	G6GAG	AGCCAT	GATCAG	TCTATT	GTCGGT
	1561	AATGA	ACAAAG	AGATGT	ATTCTG	GCATGT	CTTGCT
45	1621	TGCAGG	AATTG	6CCTG	ACAA	AGATG	ATCCAT
	1681	6GTAC	CTCAAT	TTTCA	TG6GT	AAAG	AGTTT
	1741	AG6G	CAATAA	TTGAG	TTATG	ACAA	ATGTAG
50	1801	ACTTG	AGATA	CAAG	TTTAT	GAA	TGCAT
	1861	TCTCAT	TCTC	GCATC	AG6AAA	ACAG	ATAGT
	1921	TTGTG	TTTGA	ACGT6	GT6AC	CTGG	TATTT

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      10      20      30      40      50      60
1981 AAGGGTATAAAGTTGGATGTGACTTGCCAGGGAAGTACAGAGTTGCACTGGACAAGTGATG
5 2041 CTTGGGAATTTGGTGGCCATGGAAGAAGCTGGTCATGATGTTGACCATTTACATCACCAG
2101 AAGGAATACCTGGAAGTTCCAGAAAACAAATTTCAATG6TCGTCCAAATTCCTTCAAAAGTGC
2161 TGTCTCCTGCGCGAACATGTGTGGCTTATTACAGA6TTGATGAACGCATGTCATAAACTG
10 2221 AAGATTACAGACAGACATTTGTAGTGAGCTACTACCAACAGCCAATATCGAGGAAAAGTG
2281 ACGAGAAAACCTTAAAGATTCATCATCTACAAATATCAGTACATCATCTACAAAAAATGCTT
2341 ATTACAGAGTTGATGAACGCATGTCAGAAGCTGAAAGATTACCAGACAGACATTTGTAGTG
15 2401 AGCTACTACTACCAACAGCCAATATCGAGGAGA6TGACGAGAAAACCTTGATGATTCATTAT
2461 CTACAAATATCAGTAACATTGGTCAGACTGTTGTAGTTTCTGTTGAGGAGA6AGACAAGG
2521 AACTTAAAAGATTCAACATCTGTAAGCATCATTAGTGATGCTGTTCCAGCTGAATGGGCTG
2581 ATTGCGATGCAAACGCTCTGGGGTGAAGGACTAGTCA6ATGATTGATCGATCCTTCTACGTT
20 2641 GGTGATCTCGGTCCGTG6CATGATGTCTTCAGGGTGGTAGCATTGACTGATTGCATCATAG
2701 TTTTTTTTTTTTTTTTTTAA6TATTTCTCTATGCATATTATTAGCATCCAATAAATTTAC
2761 TG6TTGTTGTACATAGAAAAAGTGCATTTG6CATGTATGT6TTTCTCTGAAATTTCCCA
25 2821 GTTTTGGT6CTTTG6CCTTTGGA6CCAAGTCTCTATATGTAATAAGAAAACTAAGAACAAT
2881 CACATATATAAAAT6TTAGTAGATTACCA .

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30 [0043] The 2909 bp long c-DNA contained in this clone was used for the next examples and is called cBE.

[0044] For the preparation of a plasmid p35s-BE, this cDNA was provided with the promoter of the 35s-RNA of the cauliflower mosaic virus as well as the polyadenylation signal of the octopine synthase gene of the Ti-plasmid pTiACH5. For this the orientation of the C-DNA coding for the branching enzyme was chosen in such a way that the coding strain will be readable (sense-orientation). The plasmid p35s-BE has a size of 13.6 kb and comprises the three fragments

35 A, B and C which were cloned into the cleavage sites of the polylinker of BIN19.

[0045] Fragment A (529 bp) contains the 35s promoter of the cauliflower mosaic virus (CaMV). The fragment contains the nucleotides 6909 to 7437 of the CaMV (Franck et al., Cell 21, 285-294). It was isolated as EcoRI-KpnI-fragment from the plasmid pDH51 (Pietrzak et al, Nucleic Acids Research 14, 5857-5868) and was cloned between the EcoRI-KpnI-cleavage position of the polylinker of the plasmid BIN 19.

40 [0046] Fragment B contains a 2909 bp cDNA fragment cBe which codes for the branching enzyme. It was cut out as HindIII-SmaI-fragment of the vector pUC 19 and was cloned into the SmaI-position of the polylinker of BIN 19 after filling-in of the Hind-III-position with DNA polymerase. For this the orientation of the cDNA was chosen in such a way that the coding strand is readable and a sense-RNA is formed. The cleavage sites BamHI/XbaI and PstI/SphI originate from the polylinker of pUC 19. The cleavage sites BamHI/XbaI/ Sall/PstI originate from the polylinker of BIN 19. The

45 two EcoRI cleavage sites located on the fragment B are internal cleavage sites of the fragment.

[0047] Fragment C (192 bp) contains the polyadenylation signal of the gene 3 of the T-DNA of the Ti-plasmid pTiACH5 (Gielen et al EMBO J. 3, 835,846), nucleotides 11749-11939, which are isolated as PvuII-HindIII fragment from the plasmid pAGV 40 (Herrera-Estrella et al (1983) Nature 303, 209-213) and were then cloned onto the PvuII cleavage

50 [0048] The plasmid p35s-BE was transferred into potatoes with the help of the agrobacterial system. After this whole plants were regenerated. Protein extracts isolated from tubers of these plants were tested for the existence of the branching enzyme using the western blot analysis. Further, tubers of these plants were tested for the content of amylose and amylopectin.

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## Example 2

### Preparation of the plasmid p35s-anti-BE and introduction of the plasmid the plant genome of potato.

[0049] In a similar manner to that described in Example 1, the plasmid p35s-anti-BE was prepared, but the orientation of the designated cDNA of the branching enzyme was inverted relative to the 35 S promotor. The plasmid p35s-anti-BE has a size of 13.6 kb and comprises the three fragments A, B and C which were cloned in the cleavage sites of the polylinker of BIN19.

[0050] Fragment A (529 bp) contains the 35s promoter of the cauliflower mosaic virus (CaMV). The fragment contains the nucleotides 6909 to 7437 of the CaMV (Franck et al. Cell 21, 285-294), and was isolated as EcoRI-KpnI-fragment from the plasmid pDH51 (Pietrzak et al Nucleic Acids Research 14, 5857-5868) and cloned between the EcoRI-KpnI-cleavage site of the polylinker of the plasmid BIN 19.

[0051] Fragment B contains the 2909 bp cDNA fragment cBE which codes for the branching enzyme. It was cut from the HindIII-SmaI-fragment of the vector pUC 19 and cloned in the SmaI-position of the polylinker BIN 19 after filling in of the HindIII-position with DNA polymerase. The orientation was chosen in such a way that the non-coding strand is readable and an anti-sense-RNA is formed. The cleavage sites SphI, PstI and XbaI, BamHI, SmaI originate from the polylinker pUC 19. The cutting positions BamHI/XbaI/SalI/PstI originate from the polylinker of BIN 19. The two EcoRI cleavage sites contained on the fragment B are internal cleavage sites of this fragment.

[0052] Fragment C (192 bp) contains the polyadenylation signal of gene 3 of the T-DNA of the Ti-plasmid pTiACH5 (Gielen et al EMBO J 3, 835-846), nucleotides 11749-11939, which were isolated as PvuII-HindIII-fragment from the plasmid pAGV 40 (Herrera-Estrella et al (1983), and which were cloned between the SphI-HindIII-cleavage position of the polylinker of BIN 19 after addition of SphI-linkers to the PvuII-cleavage position (see Fig 2).

[0053] The plasmid p35s-anti-BE was transferred into potatoes using the agrobacterial system. After this whole plants were regenerated.

[0054] Protein extracts, which had been isolated from tubers of these plants, were tested for the existence of the branching enzyme using the western blot analysis. Tubers of these plants were also tested for the content of amylose and amylopectin.

## Example 3

### Preparation of the plasmid p33-BE and introduction of the plasmid into the plant genome of the potato.

[0055] In a similar manner to that described in Example 1, the plasmid p33-BE was prepared, but replacing the 35s promoter with the promoter of the class I patatin-gene B33 (Rocha-Sosa et al EMBO J 8 23-29). The plasmid p33-Be has a size of 14.6 kb and consists of the three fragments A, B and C that were cloned into the cleavage position of the polylinker of BIN 19.

[0056] Fragment A contains the DraI-DraI-fragment (position -1512 to position +14) of the promoter region of the patatin-gene B33 (Rocha-Sosa et al EMBO J 8. 23-29), which was first of all cloned into the SacI-position of the polylinker of pUC 18. For this the overhanging 3'- end of the SacI-cleavage site had been rendered blunt by T4-DNA polymerase. After this the EcoRI-BamHI-fragment was inserted between the EcoRI-BamHI-position of the polylinker of BIN 19.

[0057] Fragment B contains the 2909 bp cDNA fragment cBE which codes for the branching enzyme. It was cut out as HindIII-SmaI-fragment from the vector pUC 19 and was cloned into the SmaI-position of the polylinker of BIN 19 after the HindIII-position was filled in with DNA polymerase. For this the orientation of the cDNA was chosen in such a way that the coding strand was readable and a sense-RNA was formed. The cleavage sites BamHI/XbaI and PstI/SphI originate from the polylinker of pUC 19. The cutting positions BamHI/XbaI/SalI/PstI originate from the polylinker of BIN 19. The two EcoRI-cleavage sites contained on the fragment B are internal cleavage sites of this fragment.

[0058] Fragment C (192 bp) contains the polyadenylation signal of gene 3 of the T-DNA of the Ti-plasmid pTiACH5 (Gielen et al EMBO J 3, 835-846, Nucleotide 11749-11939), which was isolated as PvuII-HindIII-fragment from the plasmid pAGV 40 (Herrera-Estrella et al (1983) Nature 303, 209-213) and which was cloned between the sphI-HindIII-cleavage site of the polylinker of BIN 19 after addition of SphI-linkers to the PvuII-cleavage site.

[0059] The plasmid p33-BE was transferred into Agrobacterium tumefaciens and used for the transformation of potato plants.

Example 4Preparation of the plasmid p33-anti-BE and introduction of plasmid into the plant genome of potato.

[0060] In a similar manner to that described in Example 2, plasmid p33-anti-BE was prepared but replacing the 35S-promoter with the promoter of the class I patatin-gene B33 (Rocha-Sosa et al EMBO J 8, 23-29). The plasmid p33-anti-BE has a size of 14.6 kb and consists of three fragments A, B and C which were cloned into the cleavage sites of the polylinker of BIN 19.

[0061] Fragment A contains the DraI-DraI-fragment (position -1512 to position +14) of the promoter region of the patatin-gene B33 (Rocha-Sosa et al EMBO J 8, 23-29) which was firstly cloned into the SacI-position of the polylinker of pUC 18. The overhanging 3'-ends of the SacI-cleavage site were rendered blunt by T4-DNA polymerase. After this the fragment was inserted as EcoRI-BamHI-fragment between the EcoRI-BamHI-position of the polylinker of BIN 19.

[0062] Fragment B contains the 2909 bp cDNA fragment cBE which codes for the branching enzyme. It was cut out as HindIII-SmaI-fragment from the vector pUC 18 and after filling in the HindIII-position with the DNA polymerase, it was cloned into the SmaI-position of the polylinker of BIN 19. For this the orientation of the cDNA was chosen in such a manner that the non-coding strand was readable and anti-sense-RNA could be formed. The cutting positions SphI, PstI and XbaI, BamHI, SmaI originate from the polylinker of pUC 19. The cutting positions BamHI/ XbaI/Sall/PstI originate from the polylinker of BIN 19. The two EcoRI cleavage sites which are located on the fragment B are internal cleavage sites of the fragment.

[0063] Fragment C (192 bp) contains the polyadenylation signal of the gene 3 of the T-DNA of the Ti-plasmid pTiACH5 (Gielen et al EMBO J 3, 835-846), Nucleotides 11749-11939), which had been isolated as PvuII-HindIII-fragment from the plasmid pAGV 40 (Herrera-Estrella et al (1983), Nature 303, 209-213) and which was cloned between the SphI-HindIII-cleavage site of the polylinker of BIN 19 after addition of SphI-linkers to the PvuII cleavage sites.

[0064] The plasmid p33-anti-BE was introduced in Agrobacterium tumefaciens and was used for the transformation of potato plants.

Example 5

[0065] The nucleotides 166-2909 of the 2909 bp cDNA sequence described in Example 1, that codes for the branching enzyme in the HindIII-cleavage site of the cloning vector pUC 19 were inserted into the corresponding cleavage sites of the polylinker of the cloning vector pUC 18. This makes possible a fusion of the N-end of the  $\alpha$ -peptide of the  $\beta$ -galactosidase located on the vector with a part of the branching enzyme. The functionality of the resulting fusion protein was tested in a mutant of Escherichia coli (KV 832) which is deficient in the branching enzyme (Kiel et al Gene 78, 9 - 17). Cells transformed with this construction were plated out on YT-agar plates containing 0.5% glucose. The resulting colonies were stained with Lugolscher solution. The transformed plant cells showed a yellow-red colour in contrast to the blue coloured un-transformed plant cells which indicates the branching activity of the fusion protein (Kiel et al Gene 78, 9-17). An over-production of this protein in Escherichia coli enables the use as technical enzyme.

**Claims**

1. A transgenic plant transformed with a DNA sequence comprising the coding sequence of a branching enzyme, wherein the DNA sequence contains information that causes changes in the carbohydrate concentration and the carbohydrate composition in regenerated plants, after insertion into the plant genome.
2. The transgenic plant according to claim 1, wherein said DNA sequence comprises Seq ID No. 1.
3. The transgenic plant according to claim 1 or 2, wherein said DNA sequence is fused to regulatory sequences of other genes in an inverted direction.
4. The transgenic plant according to any of claims 1 to 3, said plant producing an antisense RNA of the branching enzyme gene.
5. The transgenic plant according to any of claims 1 to 4 which is maize, barley, wheat, rice, pea, soya bean, sugar cane, sugar beet, tomato, potato or tobacco.
6. A cell of the transgenic plant according to any of claims 1 to 5 transformed with a DNA sequence comprising the coding sequence of a branching enzyme, wherein the DNA sequence contains information that causes changes

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in the carbohydrate concentration and the carbohydrate composition in regenerated plants, after insertion into the plant genome..

- 5 7. A DNA sequence comprising the coding sequence of a branching enzyme, wherein the DNA sequence contains information that causes changes in the carbohydrate concentration and the carbohydrate composition in regenerated plants, after insertion into the plant genome.
8. The DNA sequence according to claim 7, which is Seq ID No. 1.
- 10 9. Use of a DNA sequence according to claim 7 or 8 for the production of a transgenic plant cell or plant, wherein the amylose/amylopectin ratio of the starch is modified.
10. Use of a DNA sequence according to claim 7 or 8 for the production of a transgenic plant cell or plant, wherein said plant cell or plant is a commercially useful plant.
- 15 11. Use of a DNA sequence according to claim 7 or 8 for the production of a transgenic plant cell or plant, wherein said plant cell or plant is maize, barley, wheat, rice, pea, soya bean, sugar cane, sugar beet, tomato, potato or tobacco.
- 20 12. Use of a DNA sequence according to claim 7 or 8 for the production of an antisense RNA in a transgenic plant cell or plant.
- 25 13. Use of a DNA sequence according to claim 7 or 8 fused in an inverted direction to regulatory sequences of other genes for the production of an antisense RNA in a transgenic plant cell or plant.

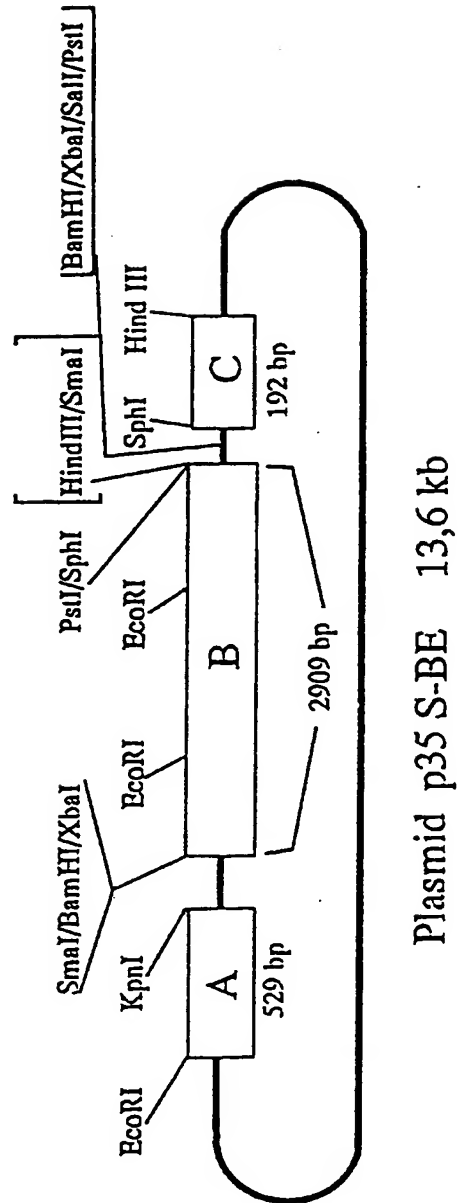


Fig. 1

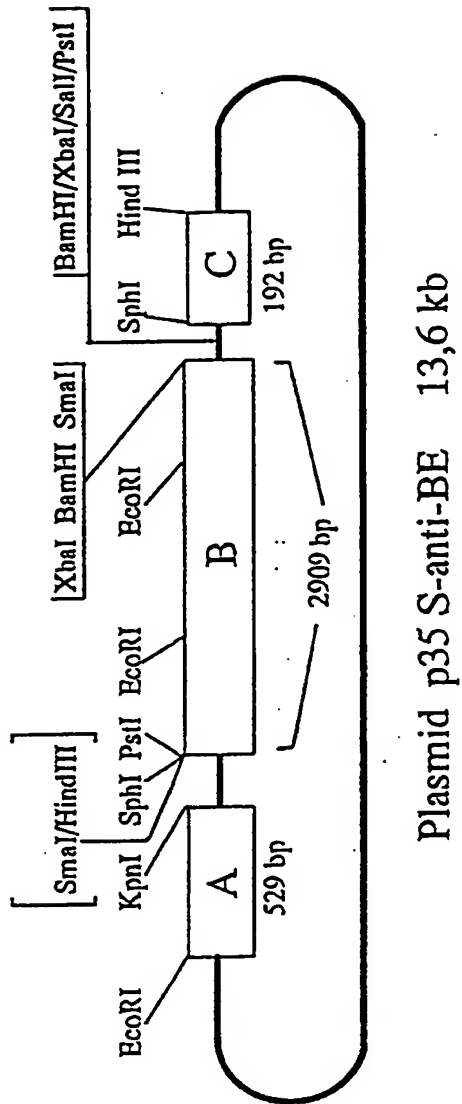


Fig. 2

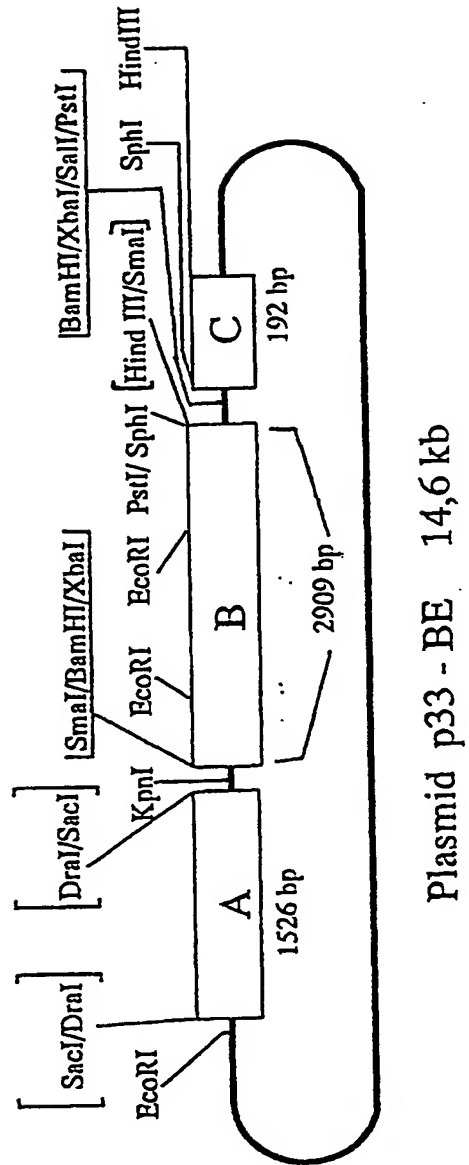


Fig. 3



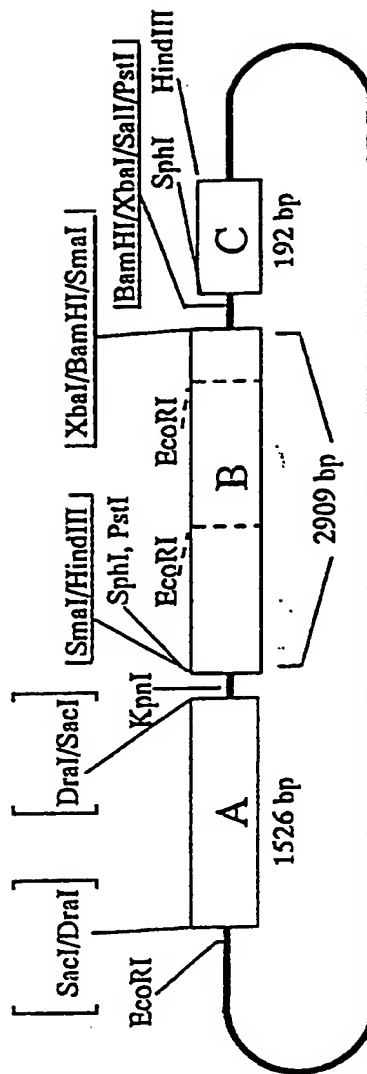


Fig. 4

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Europäisches Patentamt  
European Patent Office  
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(12)

**EUROPEAN PATENT APPLICATION**

(88) Date of publication A3:  
**28.11.2001 Bulletin 2001/48**

(51) Int Cl.<sup>7</sup>: **C12N 15/82, C12N 15/54,  
C12N 9/10, A01H 5/00**

(43) Date of publication A2:  
**30.05.2001 Bulletin 2001/22**

(21) Application number: **01100504.8**

(22) Date of filing: **11.02.1992**

(84) Designated Contracting States:  
**AT BE CH DE DK ES FR GB GR IT LI LU MC NL SE**

(30) Priority: **13.02.1991 DE 4104782**

(62) Document number(s) of the earlier application(s) in  
accordance with Art. 76 EPC:  
**92904007.9 / O 571 427**

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(54) **Plasmids containing DNA-sequences that cause changes in the carbohydrate composition in plants, as well as plant cells and plants containing these plasmids**

(57) Plasmids are described having DNA sequences that after insertion into the genome of the plants cause changes in the carbohydrate concentration and the carbohydrate composition in regenerated plants.

These changes can be obtained from a sequence of a branching enzyme that is located on these plasmids. This branching enzyme alters the amylose/amylopectin ratio in starch of the plants, especially in commercially used plants.

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## EUROPEAN SEARCH REPORT

EPO FORM 1503 03.82 (P04C01)

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## EUROPEAN SEARCH REPORT

Application Number  
EP 01 10 0504

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E	WO 92 11375 A (AMYLOGENE HB) 9 July 1992 (1992-07-09) * the whole document *	1,3-7, 9-13	
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 1 October 2001	Examiner Maddox, A
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			

EPO FORM 1503 (03.92) (P/0401)



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# EUROPEAN SEARCH REPORT

Application Number  
EP 01 10 0504

DOCUMENTS CONSIDERED TO BE RELEVANT			
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A	<p>LEIJ VAN DER F R ET AL: "EXPRESSION OF THE GENE ENCODING GRANULE BOUND STARCH SYNTHASE AFTER INTRODUCTION IN AN AMYLOSE-FREE AND A WILDTYPE POTATO (SOLANUM TUBEROSUM)"</p> <p>ABSTRACTS OF THE INTERNATIONAL CONGRESS ON PLANT CELL TISSUE AND CELL CULTURE, XX, XX,</p> <p>24 June 1990 (1990-06-24), page 177</p> <p>XP002014046</p> <p>-----</p>		
			<p>TECHNICAL FIELDS SEARCHED (Int.Cl.7)</p>
The present search report has been drawn up for all claims			
Place of search		Date of completion of the search	Examiner
THE HAGUE		1 October 2001	Maddox, A
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone</p> <p>Y : particularly relevant if combined with another document of the same category</p> <p>A : technological background</p> <p>O : non-written disclosure</p> <p>P : intermediate document</p>		<p>T : theory or principle underlying the invention</p> <p>E : earlier patent document, but published on, or after the filing date</p> <p>D : document cited in the application</p> <p>L : document cited for other reasons</p> <p>.....</p> <p>&amp; : member of the same patent family, corresponding document</p>	

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ON EUROPEAN PATENT APPLICATION NO.**

EP 01 10 0504

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